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14. ABSTRACT

This project uses a mouse model to examine the effects of rhythm disruption on the expression of genes, the growth of breast cancer xenografts and spontaneous mammary tumours. **Task 2:** Task has been completed except for some additional gene expression assays. **Task 3:** Collection of tissues from the PyMT transgenic mice has been partially completed. Second cohort of animals are currently growing under the control and shiftwork simulation conditions. **Task 4:** A viable *SCID+Clock$_{\Delta 19}$* mouse colony has been established and expanded. These mice will be used in a xenograft experiment (**Task 5**). **Task 6:** We have commenced this Task and have demonstrated partial knock-down of Bmal1 expression. Experiments are continuing to clonally expand the cell lines and conduct functional assays on them.

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INTRODUCTION

The current project uses a mouse model to examine the effect of circadian disruption (shiftwork) on genes that are relevant to the genesis and progression of breast cancer. This project arose in the light of information derived from epidemiological studies suggesting that shiftworkers have a higher incidence of breast cancer (1;2).Two recent meta-analyses have been prepared that further support the potential role of shiftwork in breast cancer (3;4). This project addresses a very simple question: Does circadian disruption contribute to this increased incidence of cancer in shiftworkers?

In recent times we have gained a good understanding of the molecular mechanisms and components that sustain circadian timing and we now know that each cell contains a circadian molecular oscillator that is tightly synchronized at a tissue specific level. Rhythmicity is synchronized with the external environment through the influence of retinally perceived light on a central oscillator located in the cells of the suprachiasmatic nucleus of the hypothalamus (5)

Recent information in the literature examining differential circadian expression of the transcriptome by using high throughput techniques such as microarray suggest that a strikingly high number of genes are directly or indirectly controlled by the cellular circadian clock (6).

Amongst these clock controlled genes are some that are directly involved in the control of cell cycle and apoptosis. Other genes are tumour suppressor genes. Indeed, in a society that is increasingly driven by a 24/7 lifestyle, where a significant proportion of the population is expected to do shiftwork, there is a high probability that critical genes involved in the regulation of cell proliferation and apoptosis are expressed at the wrong time of the circadian cycle or whose rhythmicity is disrupted.

BODY

In accordance with the STATEMENT OF WORK we have undertaken

Task 1.- Expression of clock and clock controlled genes in the mammary tissue of mice under simulated shift work conditions (rapid phase shifting of the LD cycle):
This task has been completed (see 2007 report). Data from this task are to be combined with that from Task 2 for publication. We expect to submit this in May 2008.

TASK 2 Growth and gene expression of human breast cancer cells in nude BALB/c-Foxn1^{nu} mice under simulated shiftwork conditions.
This task has been completed except for the analysis of several genes because they require the re-design of human-specific primers.
Briefly, 1 X 10^6 MCF7 human cancer cells were injected into the flank of estrogenized female nude mice. 30 mice were subjected to a simulated shiftwork protocol and 30 mice were kept under a 12L:12D lighting regime for a period of 28 days. After this period, the tumours and normal mammary tissue were dissected from the mice, the RNA was extracted, reverse-transcribed and used to assess gene expression throughout the 24 hour collection period.

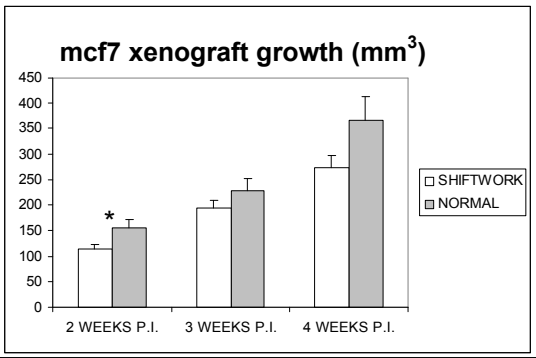

Fig 1.- MCF7 tumour growth rate in nude mice subjected to normal vs. shiftwork conditions

Tumours derived from MCF7 subcutaneous grafts in the non-shiftwork mice had a significantly larger volume at 2 week post-inoculation ($p<0.02$, two tailed), but this difference was not apparent at 3 and 4 weeks (fig 1).

<u>Clock gene expression in mammary tissue:</u>
The expression pattern of core-clock genes in the mammary tissue of mice carrying the MCF7 xenografts is shown in figure 2. Both *Bmal1* and *per2* were expressed rhythmically and in the appropriate phase, thus providing strong supporting evidence of a functional clock in mammary tissue.

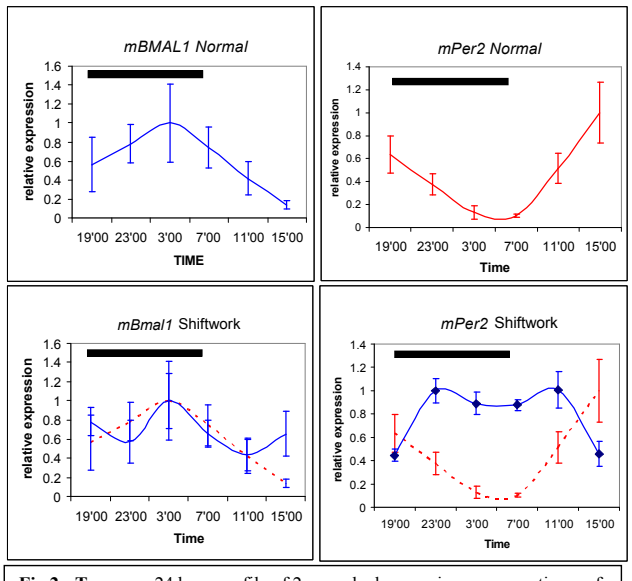

Fig 2.- Top row: 24 hour profile of 2 core clock genes in mammary tissue of mice under a 12L:12D phtoperiod. Dark bar indicates dark period. **Bottom row:** Effect of simulated shiftwork on the expression profile of these same genes. 12L12D profile superimposed for comparison (dotted line)

Clock gene expression in MCF7 tumours

We also examined core-clock genes in the MCF7 tumours using human-specific primers. As expected from the results of previous experiments we have observed considerable alteration in the expression profile of genes in the benign mammary tissue in those animals subjected to a simulated shiftwork protocol. It is particularly interesting that the rhythm of *Bmal1* appears attenuated by shiftwork.

Functional gene expression in mammary tissue

Expression of *c-myc*, *cyclin D1* and *wee1* which are putative clock controlled genes showed expression profiles that did not change significantly across 24 hours (ANOVA), but showed a trend to increased levels at the appropriate time of the circadian cycle, consistent with a level of circadian rhythm control (figure 3). These data will be subjected to further statistical analysis (eg Cosinor curve fitting). The patterns of expression were altered by the simulated shiftwork protocol, but they too require further statistical evaluation.

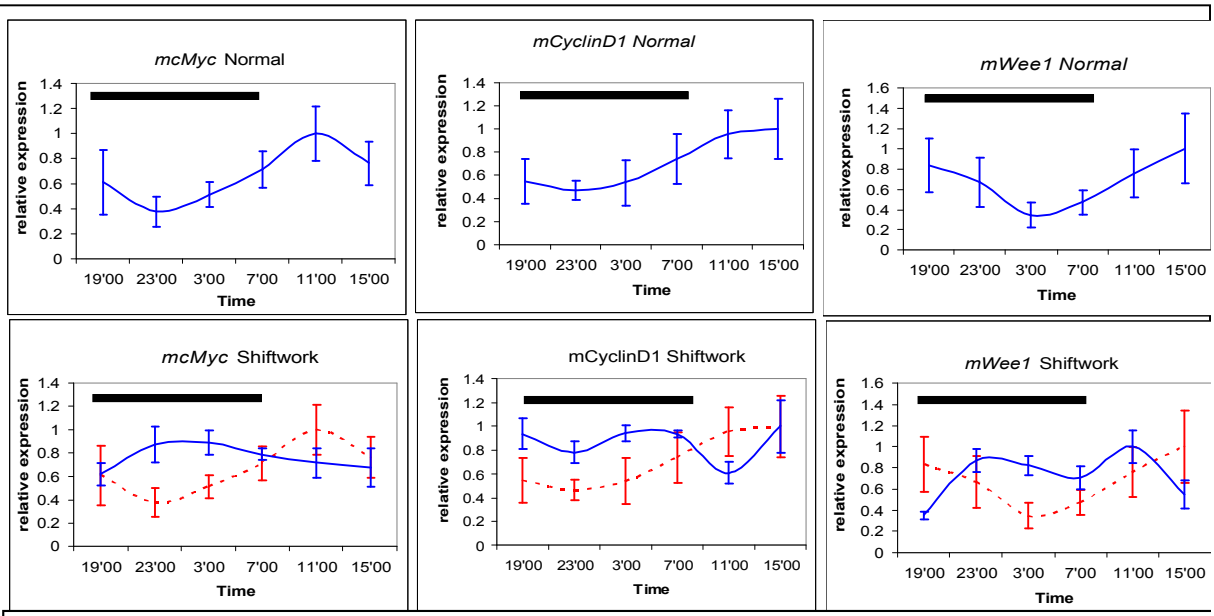

Fig 3.- Top row: 24 hour expression profiles of cell cycle genes in mouse mammary tissue under 12L:12D photoperiod. **Bottom row:** Effect of simulated shiftwork on the same genes. Profiles of 12L:12D are superimposed for comparison (dotted lines)

Clock gene expression in MCF7 xenografts

Gene expression was more variable in the MCF-7 tumours than the mammary tissue (figure 4). While the rhythm of expression of *Bmal1* was not statistically significant (ANOVA), it was highest in the late dark phase and thus similar to the time of peak expression in mammary tissue. *Rev-erbα* was rhythmically expressed with the timing compatible with it's repressive actions on *Bmal1*. In contrast, there was no suggestion of rhythmicity in *Per1* and *Per2* mRNA expression suggesting that the time-keeping system is dysfunctional in the MCF7 tumour cells in animals kept in a normal photoperiod. The shiftwork simulation further dampened the *Bmal1* and *Rev-erbα* excursions and did not improve the patterns of expression of *per1* and *per2*. These results support the human *in situ* data that reports a down-regulation in the expression of *Per1 and Per2*.

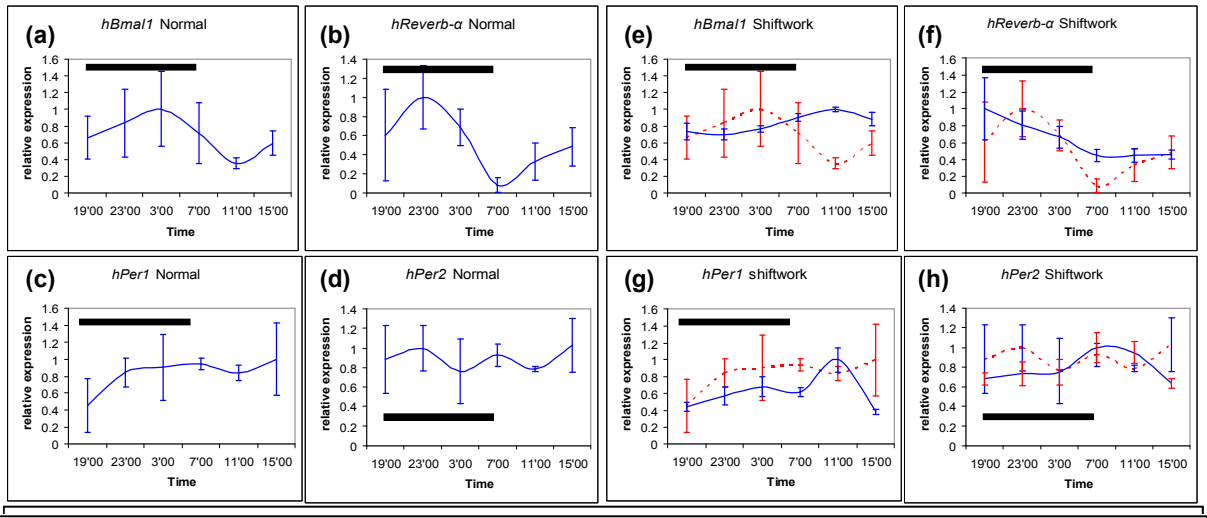

Figure 4.-**Panels a-d**: 24 hour expression profiles of clock genes in MCF7 xenografts in mice maintained on a 12L:12D photoperiod. **Panels e-h**: Effect of the shiftwork simulation on the same genes. Profiles from the mice in 12L:12D are superimposed for comparison (dotted lines)

Functional gene expression MCF7 xenografts

The expression of *cMyc* or *Wee1* were analysed in xenografts in animals kept on a 12L:12D photoperiod and the shiftwork simulation. Expression of both genes was not rhythmic and the shiftwork simulation did not alter the pattern of expression. Other genes are currently under investigation.

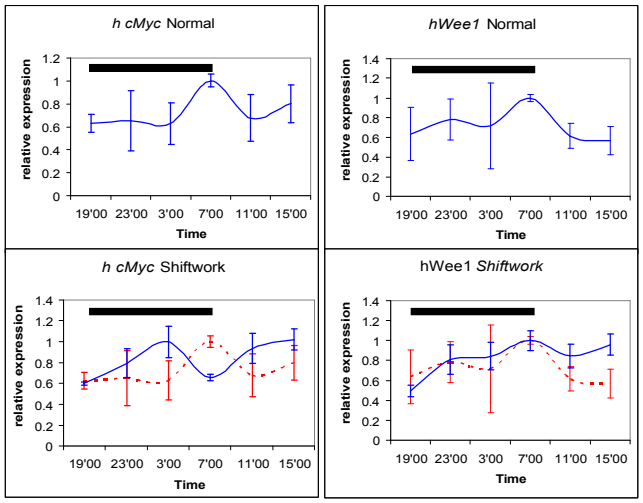

Fig 5.- Top row: 24-hour profile of 2 cell cycle genes in MCF7 tumours grafted in to mice under 12L:12D. **Bottom row:** 24 hour profile of same genes in mice subjected to simulated shiftwork

TASK3: Growth and progression to malignancy of mammary tumours in PyMT transgenic mice under simulated shiftwork conditions.

This experiment is progressing well. Due to the difficulties in breeding sufficient mice to complete all arms of the experiment, it has been divided into 2 parts with equal numbers of experimental and control mice in each run. The first part has been completed and preliminary data analyses conducted. Animals were killed at 14 weeks of age after 9 weeks on the shiftwork simulation protocol to determine total tumour burden, primary tumour weight in the 4th mammary pad and the degree of metastasis to the lung.

Figure 6 shows that mice exposed to the shiftwork simulation had a significantly larger tumour burden than mice maintained on the normal photoperiod (Fig 6A; P<0.028, one tailed). The shiftwork-simulated mice also had significantly larger tumours in the 4th mammary pad than controls (Fig 6B; P<0.03, two tailed,) These results show that shiftwork impinges negatively on the growth rate of mammary tumours.

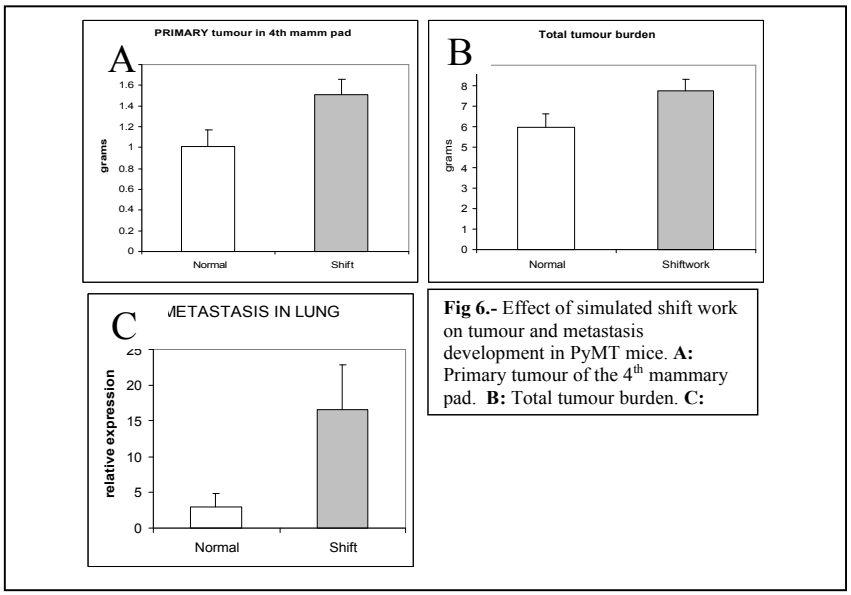

Fig 6.- Effect of simulated shift work on tumour and metastasis development in PyMT mice. **A:** Primary tumour of the 4th mammary pad. **B:** Total tumour burden. **C:**

Using this PyMT model we have also examined the degree of metastasis to the lung using Real Time RT-PCR to assess the number of cells expressing the transgene in lung. Our preliminary data indicates that there is a significantly higher level of metastasis to the lung in the mice that were in the shiftwork simulation (Figure 6C; P<0.03, one tailed).

TASK 4: Development of an immunodeficient *Clock*$^{\Delta 19}$mutant mouse strain.

As previously reported, we successfully generated mice homozygous for both the *Foxn1*^{nu} and *Clock*$^{\Delta 19}$ mutations, however, the offspring of this cross had a lactation deficit and the colony was not viable. We have now succeeded in crossing the *Clock*$^{\Delta 19}$ mutation onto a severe combined immunodeficient (SCID) mouse background. The colony is currently being expanded to produce sufficient mice to test the effects of a non-rhythmic host on MCF7 xenograft growth, etc.

TASK 5: Growth and gene expression of human breast cancer cells in immunodeficient *SCID+Clock*$^{\Delta 19}$ mutant mice

Now that we have this double mutant mouse available, this experiment will commence in mid-March.

TASK 6: Effect of disruption of clock gene function with small interfering mRNA on the growth and gene expression of human breast cancer cell lines *in vitro* and *in vivo.*

We have down-regulated the master clock gene *BMAL1* in the MCF7 estrogen dependent human cancer cell line and MCF10A "normal" human breast cell line using an siRNA approach. The down-regulation has been confirmed at the RNA level using Real Time RT-PCR. These cells have been expanded and frozen. The transfectant line #3 has a 60% decrease in *Bmal1* expression (figure 7). We are now proceeding with the clonal selection of cells to investigate the effects of clock disruption on proliferation and apoptosis.

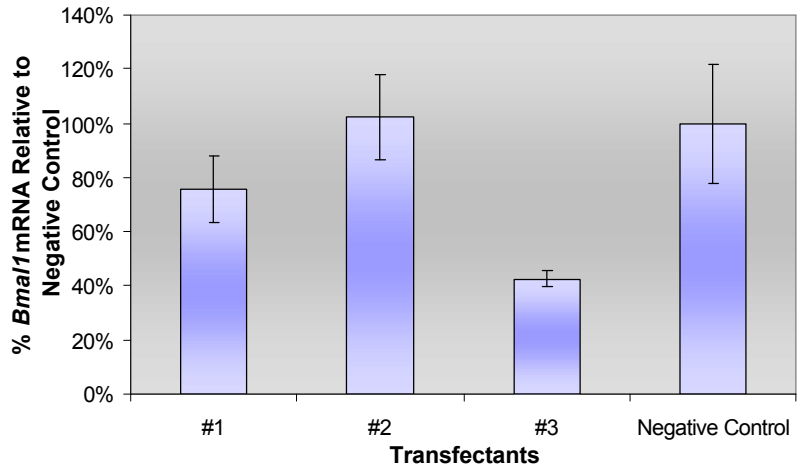

Fig 7.- One of the 3 commercially available shRNA constructs was successful in knocking down *Bmal1* expression as determined by semi-quantitative PCR

KEY RESEARCH ACCOMPLISHMENTS

Task 1: Manuscript in preparation

Task 2: Task has been completed except for some additional gene expression assays.

Task 3: Collection of tissues partially completed. Second cohort of animals are currently growing under the control and shiftwork simulation conditions.

Task 4: A viable *SCID+Clock*$^{\Delta 19}$ mouse colony has been established and expanded.

Task 6: We have commenced this task and have demonstrated partial knock-down of *Bmal1* expression. Experiments are continuing to clonally expand the cell lines and conduct functional assays on them.

REPORTABLE OUTCOMES

Clock genes are expressed in MCF7 xenografts, but there appears to be aberrant expression of *per1* and *per2* such that there is no discernible rhythm of expression of these genes despite rhythmicity of *Bmal1* and *Rev-erbα*. When the xenografts developed in mice subjected to the shiftwork simulation all 4 genes studied so far did not display rhythmic gene expression.

Our preliminary results of the impact of rhythm disruption (shiftwork simulation) in PyMT transgenic mice provides exciting evidence that the primary tumour size, total tumour burden and metastases are increased.

CONCLUSIONS

We believe that the results that we have obtained so far in this project provide strong experimental evidence that clock genes and circadian rhythmicity play a functional role in mammary tissue. Further, disruption of rhythmicity through rapid photoperiod change simulating shiftwork, impacts on gene expression of tumour xenografts (without apparently altering growth rate), and the development of endogenous mammary tumours.

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